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Original Article

Screening of Biosurfactant-producing Bacteria from Symbiotic Microbes with Gastropods in the Persian Gulf

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ABSTRACT

Introduction: Biosurfactants or surface-active compounds with amphiphilic molecular structures, including a hydrophilic and a hydrophobic domain, are produced by microorganisms. These compounds increase the biodegradation of hydrocarbons in the environment due to their ability to emulsify hydrocarbon-water mixtures. This study was conducted to isolate and characterize biosurfactant-producing bacteria from the samples of Gastropods.
Materials and Methods: The gastropod samples were collected from oil-contaminated sites in the Persian Gulf, Middle East. Biosurfactant-producing strains were isolated from these samples. The biosurfactant production ability was analyzed using Drop Collapse TEST, oil spreading test, emulsification activity test, and BATH test.
Results: In total, 11 biosurfactant-producing strains were isolated. Two isolates with higher growth rates and biosurfactant production ability were selected for further studies. The best isolates were identified as *Halomonas* sp. isolate BHA16 and *Vibrio alginolyticus* isolate BHA 17 based on molecular analysis. Gas chromatography analysis of remaining crude oil confirmed that these strains could degrade to 51.44 % and 67.58% of crude oil, respectively.
Conclusion: The results of this study indicated the surfactant activity of the bacterial strains isolated from Gastropods had a good potential for the biodegradation of crude oil and could be used for the cleanup of oil-contaminated marine environments.

1. Introduction

The Persian Gulf is an important marine environment in the south of Iran, the pathway for about 60 percent of the marine transported oil in the world. The Persian Gulf was polluted with crude oil during the 1991 Gulf war[1]. Oil spills in the sea have become a global problem in industrialized and developing countries. It is one of the most dangerous pollution factors known today[2]. Oily wastewater contains toxic substances, such as phenols, petroleum hydrocarbons, and polyaromatic hydrocarbons, inhibiting plant and animal growth and being mutagenic and carcinogenic to people[3,4]. It can be a threat to the environment, necessitating monitoring and cleanup of pollution to protect the environment[5]. One of the strategies to enhance the cleanup is bioremediation which degrades environmental pollution by microorganisms[6]. Many bacteria from contaminated areas can produce biosurfactants. Biosurfactants produced by hydrocarbon-degrading bacteria with amphiphilic molecular structure (including a hydrophilic and a hydrophobic domain) can be emulsified hydrocarbon-water mixtures, which enables them to grow on the oil droplets and increase the biodegradation of hydrocarbons in the environment[7,8]. Several approaches measure the surface activity of biosurfactants, including hemolysis in blood agar, oil spreading, drop collapse test, emulsification activity, and BATH test[9-11]. Therefore, this study aimed to identify biosurfactant-producing bacteria from gastropod samples collected from oil-contaminated areas in the Persian Gulf. Additionally, the degradation capacity of these strains was examined.

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2. Materials and Methods

2.1. Sampling

To isolate biosurfactant-producing bacteria, gastropod samples were collected from oil-contaminated sites in the Persian Gulf in August 2016. This station was located on Qeshm Island, Iran (HA: Haghni Harbor). Gastropods were collected by a knife from a depth range of 5-15 meters. Collected samples were transported on ice to the laboratory within 6 hours. Gastropod samples were washed with marine water twice[12]. Finally, the solution of gastropods was used for subsequent studies.

2.2. Isolation of biosurfactant-producing bacteria

The ONR7a medium was supplemented with 1% (v/v) of crude-oil (Iranian light crude oil) as the sole carbon source and energy used to isolate biosurfactant-producing strains of bacteria. ONR7a contained (per liter of distilled water) 40g of NaCl, 11.18 g of $MgCl_2.6H_2O$, 3.98 g of Na_2SO_4, 1.46 g of $CaC1_2.2H_2O$, 1.3 g of TAPS0 {3-[N tris(hydroxymethyl) methylamino]-2 hydroxypropanesulfonic acid}, 0.72 g of KCl, 0.27 g of NH_4Cl, 89 mg of $Na_2HP0_4.7H_20$, 83 mg of NaBr, 31 mg of $NaHCO_3$, 27mg of H_3BO_3, 24 mg of $SrCl_2.6H_20$, 2.6 mg of NaF and 2 mg of $FeCl_2.4H_20$. For solid media, Bacterial Agar (15 g/l) was added[12]. All materials were purchased from Merk Co., Germany.

A gastropod solution (5 ml) was added to Erlenmeyer flasks containing 100 ml of medium, and the flask was incubated for 7 days at 30°C on a rotary shaker (180 rpm, INFORS AG). Then, 5 ml was transported to a fresh medium. After a series of four subcultures, inoculum from the flask was streaked out, and phenotypically different colonies on ONR7a agar were purified. Phenotypically, different colonies obtained from the plates were transferred to fresh medium with and without crude oil to eliminate autotrophs and agar-utilizing bacteria. The procedure was repeated, and the only isolates exhibiting pronounced growth on crude oil were stored in stock media with glycerol at -20°C for further characterization[13].

2.3. Screening methods

2.3.2. Drop collapse test (qualitative screening)

A drop of the culture supernatant was placed carefully on an oil-coated glass slide and observed after one minute. In case the drop of supernatant collapses and spreads on the oil-coated surface, it signifies the presence of biosurfactant (positive). However, if the drop remains after one minute, it was documented as negative. This test was simultaneously carried out on distilled water as control[14].

2.3.1. Oil spreading test (quantitative screening)

To employ the oil spreading technique, 50 ml of distilled water was added to a large petri dish (25 cm diameter), followed by the addition of 20 µl of crude oil to the surface of the water. In the next step, 10 microliters of cell-free broth of MB culture (Centrifuged at 10000 rpm for 10 minutes.) were then added to the surface of the oil. The diameter of the clear zone on the oil surface was measured. Moreover, the diameters of triplicate samples from the same culture of each strain were determined[15].

2.3.3. Emulsification test (quantitative screening)

The emulsification activity (E_{24}) was determined by the addition of hexadecane to the same volume of cell-free culture broth. After mixing with a vortex for 2 minutes and leaving to stand for 24 hours, the E_{24} index was measured as a percentage of the height of emulsified layer (in millimeters) divided by the total height of the liquid column (in millimeters)[16].

2.3.4. Bacterial adherence to hydrocarbons (quantitative screening)

Bacterial cells were suspended in the saline solution to reach an optical density at 600 nm (OD600 nm) of 1.0. OD600 was measured using a UV-visible spectrophotometer (Shimadzu UV-160, Japan). An aliquot of the cell suspension (4 ml) was mixed with 0.2 ml of n-hexadecane in a test tube (16×150 mm) by vortexing for 1 minute, and then the mixture was allowed to separate for 1 hour. The OD600 of the aqueous phase was then determined. The degree of hydrophobicity was expressed as the percentage of cells removed from the aqueous phase[17].

2.4. Identification of the isolates

An analysis of 16S rRNA was performed to characterize the isolated strains taxonomically. Total DNA extraction of bacterial strains was performed with the CTAB method. PCR amplification of 16S rRNA genes was performed using the general bacteria primer 27F (5-AGAGTTTGATCCTGGCTCAG-3) and universal reverse primer 1492R (5-TACGYTACCTTGTTACGACTT-3[13]. The amplification reaction was performed in a total volume of 25 µl consisting of 2mM $MgCl_2$ (1µl), 10X PCR reaction buffer (200 mM Tris; 500 mM KCl) (2.5 µl), 2mM each dNTP (2 µl), 0.15 mM each primer (1µl), 1U (0.5 µl) taq DNA polymerase (Qiagen, Hilden, Germany), and 2 µl of template DNA (50 p). The distilled water was added for the remaining reaction (15 µl). Amplification for 35 cycles was performed in a thermal cycler GeneAmp 5700 (PE Applied Biosystem, Foster City, CA, USA). The temperature profile for PCR was kept at 94°C for 5 minutes, 94°C for 1 minute, 54°C for 1 minute, 72°C for 1 minute, 30 cycles; then 72°C for 10 minutes, and finally storage at 4°C. The 16S rRNA amplified was sequenced with a Big Dye terminator V3.1 cycle sequencing kit on an automated capillary sequencer (model 3100 Avant Genetic Analyzer, Applied Biosystems). Similarity rank from the Ribosomal Database Project (RDP) and FASTA Nucleotide Database Query were used to determine partial 16S rRNA sequences to estimate the degree of similarity to other 16S rRNA gene sequences. Analysis and phylogenetic affiliates of sequences

were performed as previously described protocols[18].

2.5. Measure crude oil degradation with Gas Chromatography

Crude oil degradation was accurately estimated by GC-FID. The residual crude oil extracted in each sample. For the extraction protocol, the same volume of DCM was added to each flask, and residual crude oil was extracted and then treated with anhydrous sodium sulfate (Na_2SO_4) to remove residual water. Extracts were concentrated by separating funnel[19]. Analyses were performed using GC-FID (Varian 3800 model, USA) equipped with a SE-54 capillary column (25 m × 0.32 mm × 0.1 µm) and flame ionization detector (FID). Helium was used as the carrier gas (30 ml/minute). The oven was programmed as100ºC (1 minute), then increased to 300ºC (2 minutes) at a rate of 30ºC min-1. The samples were quantified according to previously described protocols[19].

3. Results

3.1. Identification of gastropods

Some gastropod samples were collected from the Persian Gulf. These gastropods were identified according to the standard key. The results of identification confirmed that these gastropods were related to *Haustrum scobina* genus. Figure 1 shows the morphology of these gastropods.

Figure 1. The morphology of collected gastropods from the Persian Gulf

3.2. Isolation and identification of bacteria

A total of 11 biosurfactant-producing bacteria were isolated from enrichment cultures that were incubated at 30°C for two weeks. All of the isolated strains were tested to check the abilities of biosurfactant production.

Then, two strains had the best biosurfactant production, compared to other strains, and were selected for further study (Table 1). Molecular identification of isolates was performed by amplification and sequencing the 16S rRNA gene sequencing and comparing them to the database of 16S rRNA sequences. The molecular identification of strain HA 2 and strain HA 4 indicated that these strains belonged to *Halomonas sp.* isolate BHA 16 (98%) and *Vibrio alginolyticus* isolate BHA 17 (98%). The phylogeny trees of these strains are illustrated in Figure 2. The sequences of

Table 1. Biosurfactants screening tests

Isolate	Drop collapse Test	Clearing zone (mm)	E24 (%)	BATH (%)
HA1	+	6mm	0	3
HA2	+	15mm	31.6	10
HA3	+	3mm	0	5
HA4	+	13mm	58.4	16.5
HA5	+	3mm	7.8	5.2
HA6	+	5mm	9.1	8.7
HA7	+	5mm	1.3	0
HA8	+	2mm	5.2	0
HA9	+	7mm	8.1	0
HA10	+	5mm	3.7	0
HA11	+	6mm	11.3	3

Abbreviation used: + = positive, - = negative

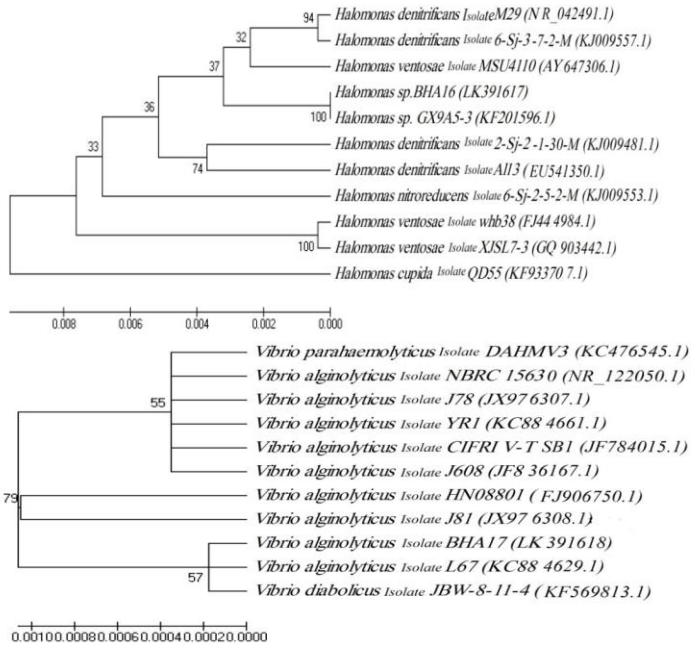

Figure 2. Phylogenetic tree of 16S rDNA sequences of the HA2 and HA4 isolates obtained from the Persian Gulf. The tree was constructed using sequences of the comparable region of the 16S rDNA gene sequences available in public databases. Neighbor-joining analysis using 1,000 bootstrap replicates was used to infer tree topology. The bar represents 0.1% sequence divergence

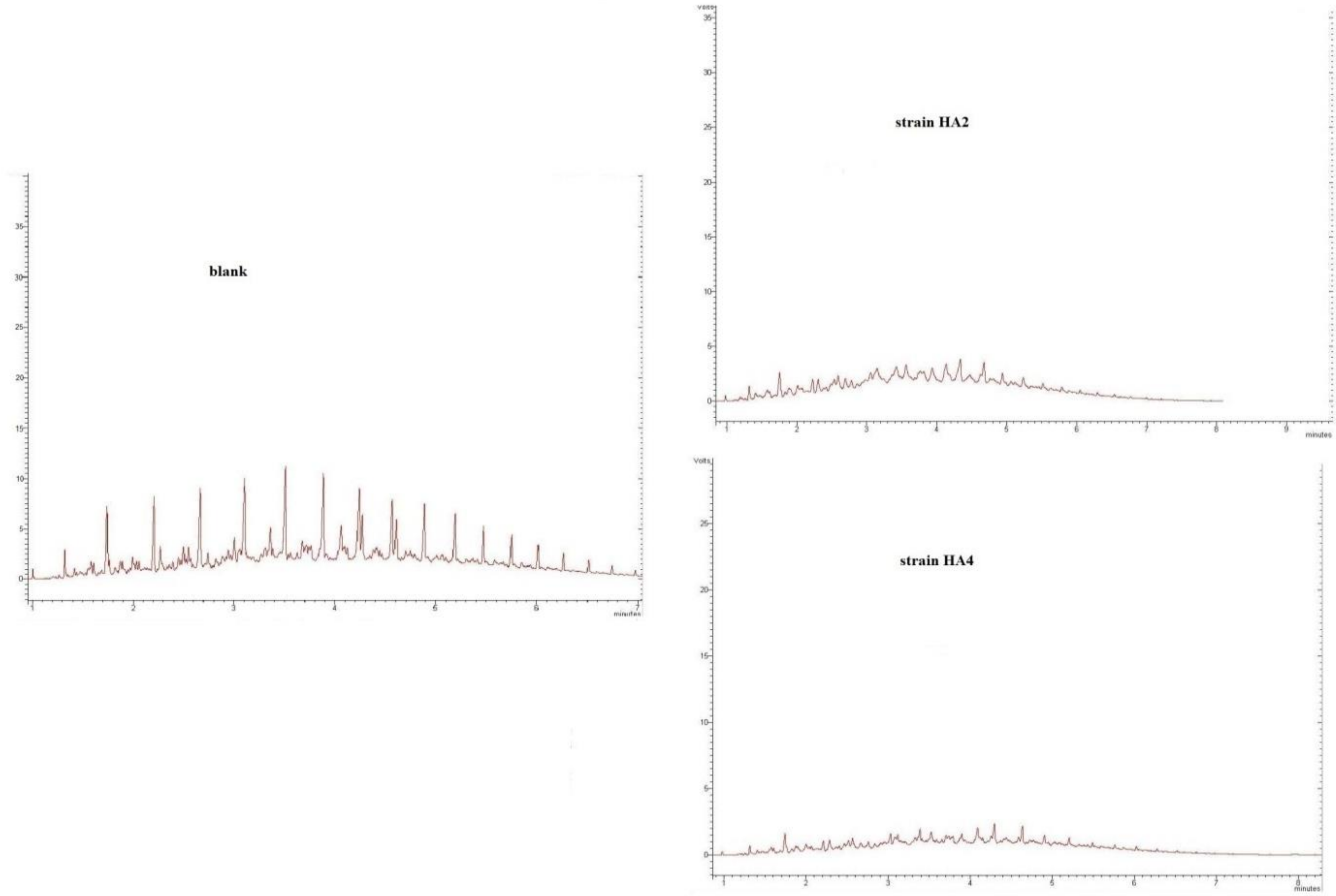

Figure 3. Gas chromatographic profiles of residual hydrocarbons of crude oil in control (blank) and strains (HA2 and HA4)

HA 2 and HA 4 strains were submitted to the Genetic sequence database at the National Center for Biotechnical Information (NCBI). Gene Bank IDs of these strains in NCBI were LK391617 and LK391618.

3.4. Growth rate, crude-oil removal

Isolated strains were grown in 1% crude oil for 15 days with shaking. After 15 days, the crude oil biodegradation levels were monitored using the GC-FID method. An analysis of the remaining crude oil by Gas Chromatography (GC) confirmed that HA2 and HA4 strains could degrade to 51.44 % and 67.58% of crude oil, respectively. Figure 3 shows the GC-FID chromatograms for these strains, compared to the blank. As can be seen, the almost peaks in the crude oil decreased dramatically in these strains.

4. Discussion

Many methods can be used for screening biosurfactant-producing marine bacteria. Kumar et al. detected biosurfactant-producing marine bacteria by some methods, such as hemolytic assay, modified drop collapse, tilted glass slide, emulsification index, oil spread method, and blue agar plate[20]. These researchers found that a single method does not suffice to identify all types of biosurfactant and efficient detection of potential biosurfactant producers, for which a combination of various screening methods are required[21,22]. In the current study, 11 biosurfactant-producing strains were isolated from gastropod samples collected from oil-contaminated areas in the Persian Gulf by qualitative screening. The drop collapse test used in this study was a qualitative screening that limited the number of samples. The potential of biosurfactant production was confirmed by quantitative screening, such as the oil spreading test, E24, and BATH test. *Halomonas sp*. and *Vibrio alginolyticus* had the best production of biosurfactant, compared to other strains, and they had a clearing zone of 15mm and 13mm, an emulsification capacity of 31.6% and 58.4%, and BATH of 10% and 16.5%, respectively. Moreover, the removal efficiency of crude oil was examined as the maximum values of hydrocarbon-degrading bacteria related to *Halomonas sp* and *Vibrio alginolyticus* at 51.44 % and 67.58%, respectively. Biosurfactant compounds increased the biodegradation of hydrocarbons in the environment due to their ability to emulsify hydrocarbon-water mixtures. The findings of this study were in accordance with that of Hassanshahian et al., who reported a positive relationship between the production of biosurfactant and hydrocarbon biodegradation[23].

5. Conclusion

The surfactant activity of the *Halomonas sp*. and *Vibrio alginolyticus* isolated from Gastropods has a good potential for the biodegradation of crude oil. It can be used for the cleanup of oil-contaminated marine environments. Furthermore, this study recommend these strains as substrates for the large-scale production of biosurfactants.

Declarations

Competing interests

The authors declare that they haven't competing interests.

Authors' contribution

All authors conceived and designed the study. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

Ethical considerations

The authors checked for plagiarism and consented to the publishing of the article. The authors have also checked the article for data fabrication, double publication, and redundancy.

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